

## Down-regulation of MHC class I by the Marek's disease virus (MDV) UL49.5 gene product mildly affects virulence in a haplotype-specific fashion

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### ABSTRACT

Marek's disease is a devastating neoplastic disease of chickens caused by Marek's disease virus (MDV). MDV down-regulates surface expression of MHC class I molecules, although the mechanism has remained elusive. MDV harbors a UL49.5 homolog that has been shown to down-regulate MHC class I expression in other *Varicelloviruses*. Using *in vitro* assays, we showed that MDV pUL49.5 down-regulates MHC class I directly and identified its cytoplasmic tail as essential for this function. *In vivo*, viruses lacking the cytoplasmic tail of pUL49.5 showed no differences in MD pathogenesis compared to revertant viruses in highly susceptible chickens of the B<sup>19</sup>B<sup>19</sup> MHC class I haplotype, while there was a mild reduction in pathogenic potential of the deletion viruses in chickens more resistant to MD pathogenesis (MHC:B<sup>21</sup>B<sup>21</sup>). We concluded that the pathogenic effect of MHC class I down-regulation mediated by pUL49.5 is small because virus immune evasion possibly requires more than one viral protein.

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### Introduction

Marek's disease (MD) is a lymphoproliferative disease of chickens that affects most visceral organs and peripheral nerves. MD is caused by a highly contagious, cell-associated, oncogenic alphaherpesvirus called Marek's disease virus (MDV), also known as gallid herpesvirus 2 (GaHV-2). Like other herpesviruses, MDV has both a lytic and latent phase of infection. MDV initially infects B lymphocytes where it replicates lytically, which is followed by infection of activated T lymphocytes. Latency is established predominantly in CD4<sup>+</sup> T lymphocytes, and lymphomas usually develop in susceptible chickens after 3 weeks post-infection (Calnek, 2001).

Escape of immune surveillance is crucial for the development and maintenance of the latent stage of infection and completion of the herpesvirus life cycle following reactivation and transmission to sentinels. Interference with major histocompatibility complex (MHC) proteins is a common mechanism targeted by a number of mammalian alphaherpesviruses to evade this important immune surveillance. For example, herpes simplex virus type 1 (HSV-1) down-regulates MHC class I surface expression by interfering with peptide loading via a direct interaction of the viral protein ICP47 with the transporter associated with antigen presentation (TAP) (Fruh et al., 1995; Hill et al., 1995). Other alphaherpesviruses like equine herpesvirus type 1 (EHV-1),

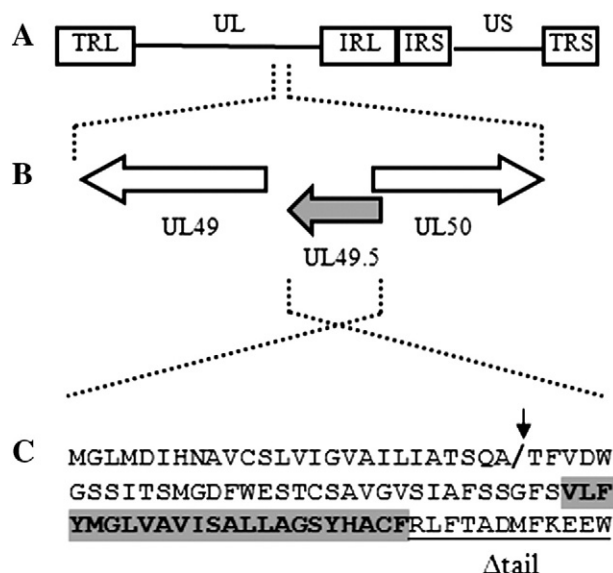
bovine herpesvirus type 1 (BHV-1), and pseudorabies virus (PRV) also down-regulate MHC class I surface expression, but do not encode an ICP47 homolog. It has been shown that the gene products of the UL49.5 orthologs will also mediate MHC class I down-regulation, at least in the case of BHV-1 and PRV, by blocking peptides transported by TAP. Simultaneously, the BHV-1 UL49.5 product induces degradation of TAP, the latter function being linked to the cytoplasmic tail of the pUL49.5 (Koppers-Lalic et al., 2003, 2005). Subsequently, it was shown that the UL49.5 proteins (pUL49.5) of EHV-1 (gene 10) and PRV, which is glycosylated in this virus and referred to as glycoprotein N (gN), also inactivate TAP, while the varicella-zoster virus (VZV) pUL49.5 homolog encoded by ORF9A does not (Koppers-Lalic et al., 2005).

It has been previously shown that MDV down-regulates cell surface expression of MHC class I proteins during active, but not latent infection of chicken cells (Hunt et al., 2001). MDV does not contain an ICP47 homolog but harbors a UL49.5 homolog, which represents a small type 1 transmembrane protein that is encoded by a gene located between the UL49 and UL50 open reading frames (Fig. 1). In contrast to other alphaherpesviruses, the UL49.5 product and its complex partner, glycoprotein M, were shown to be essential for cell-to-cell spread of MDV *in vitro* (Tischer et al., 2002).

In this report, we assessed the contribution of the MDV pUL49.5 during down-regulation of MHC class I surface expression and pathogenesis of MD. We were able to show that pUL49.5 and, more specifically, its short cytoplasmic tail are clearly responsible for significant down-modulation of MHC class I on the surface of transfected and infected cells. Interestingly, inability to down-

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**Fig. 1.** Schematic representation of the MDV genome, location of UL49.5, and the sequence of MDV pUL49.5. (A) Genomic structure of MDV showing the repeat (TRL, IRL, TRS, and IRS) and unique (UL and US) regions. (B) UL49.5 is located between UL49 and UL50 coding in the leftward direction. (C) Protein sequence of MDV pUL49.5 indicating signal cleavage site (arrow), transmembrane domain (gray highlight), and peptides deleted at the cytoplasmic tail ( $\Delta$ tail) for generation of pUL49.5 $\Delta$ tail expression plasmids and mutant viruses.

regulate MHC class I only moderately altered the pathogenic potential and virulence of MDV mutants lacking the activity and the effect proved to be specific for a certain MHC class I haplotype.

## Results

### Down-regulation of MHC class I surface expression by MDV pUL49.5

The UL49.5 gene of MDV was modified by insertion of the coding sequences for a FLAG epitope after the predicted signal peptide and was cloned into the mammalian expression vector pcDNA3.1. The resulting recombinant plasmid was transiently transfected into the chicken B cell line RP9 and MHC class I surface expression and FLAG-tagged pUL49.5 were analyzed by flow cytometry at 24 h after transfection. Flow cytometry analysis (Fig. 2A) showed two populations of cells, one expressing high levels of MHC class I on the surface and the other (~20%) expressing significantly lower levels of surface MHC class I (red line indicates cells expressing pUL49.5). MHC class I surface expression on cells transfected with empty vector pcDNA3.1 was unaffected (dashed lines), while the population of RP9 cells reactive with the FLAG antibody (red line) showed a clear down-regulation of MHC class I on the surface (red arrow) of approximately 300 mean channels of fluorescence (MCFs). As a negative control, a plasmid expressing the HSV-1 pUL49.5 did not down-regulate MHC class I surface expression (Fig. 2B).

It was previously reported that the cytoplasmic tail of pUL49.5 mediates down-regulation of MHC class I surface expression by targeting TAP for degradation in the ubiquitin–proteasome pathway (Koppers-Lalic et al., 2005). We therefore investigated whether the MDV pUL49.5 cytoplasmic tail was involved in MHC class I down-modulation. A plasmid expressing pUL49.5 that lacked the cytoplasmic tail (49.5 $\Delta$ tail) (Fig. 1C) was tested for its ability to alter MHC class I surface molecules upon transient expression. Flow cytometry analyses showed that MHC class I surface expression was unaltered in RP9 cells transfected with the truncated pUL49.5 expression vector and was virtually indistinguishable from cells transfected with either empty vector or the HSV-1 pUL49.5 expression vector (Fig. 2C).

Next, RP9 cell lines stably expressing FLAG-tagged MDV pUL49.5 were generated and surface expression of classical and non-classical

MHC class I as well as MHC class II proteins was examined using flow cytometry. While classical, peptide-presenting MHC class I was down-regulated by 260 MCFs in cells expressing MDV pUL49.5 (Fig. 2D), no effect on cell surface expression of non-classical MHC class I (Fig. 2E) or MHC class II was observed (Fig. 2F). These results indicated that MDV pUL49.5 by itself, without the need for additional MDV gene products, is capable of specifically down-modulating expression of classical, peptide-presenting MHC class I surface molecules on the cell surface. Furthermore, the results confirmed that the cytoplasmic tail of pUL49.5 mediates the observed down-regulation.

### Construction and growth properties of MDV $\Delta$ 49.5tail mutants

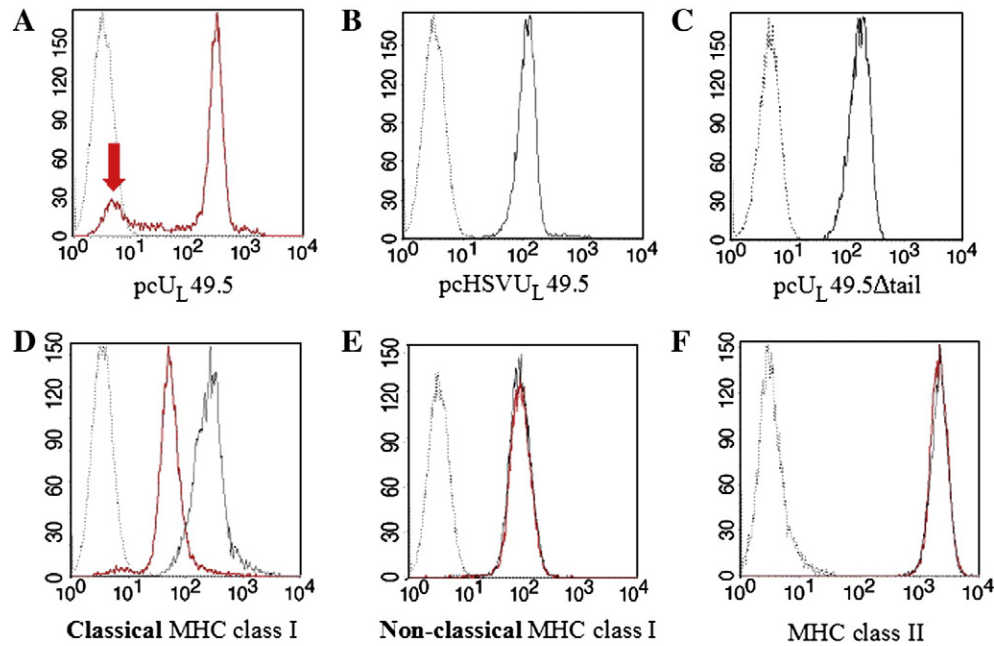
Based on the above findings and recently published reports on the ability of the pUL49.5 cytoplasmic tail of members of the *Varicelloviruses* in blocking TAP function (Koppers-Lalic et al., 2005), we constructed two independent recombinant viruses (Table 1, 1130 and 1131) based on the infectious BAC20 clone that had been derived from an avirulent MDV (Schumacher et al., 2000). The engineered recombinants lacked the amino acids constituting the cytoplasmic tail of MDV pUL49.5 (Fig. 1C). The two independent clones were assessed for their *in vitro* growth properties and found to be virtually identical to wild-type v20 (v1084) with respect to plaque sizes induced in primary chicken embryo cell (CEC) cultures (Fig. 3A) and multi-step growth kinetics in CEC (Fig. 3B). These results clearly demonstrated that the deletion of the cytoplasmic tail of pUL49.5 did not have any negative effect on virus replication. This observation was in stark contrast to that with the v20 $\Delta$ 49.5 virus, which was unable to grow in cultured cells (Tischer et al., 2002).

### Down-regulation of MHC class I surface expression by MDV is largely dependent on expression of an intact UL49.5 gene product

In order to test the role of the MDV pUL49.5 cytoplasmic tail in down-regulation of MHC class I surface expression, an *in vitro* assay was used. The immortalized chicken fibroblast cell line OU2, which constitutively expresses high levels of surface MHC class I, was infected with v1084, v1130, or v1131. At various times after infection, MHC class I surface expression was analyzed using flow cytometry. Consistent with earlier observations of MDV-induced down-regulation, approximately 75% of v1084-infected cells exhibited low levels of MHC class I surface expression (Fig. 3C). In contrast, only 32% and 24% of infected cells expressed low levels of surface MHC class I molecules in the v1130- and v1131-infected cultures, respectively, showing that down-regulation of MHC class I was greatly reduced with viruses lacking the pUL49.5 cytoplasmic tail. From these results we concluded that the MDV pUL49.5 is at least partially responsible for down-regulation of MHC class I on the cell surface in a virus context.

### Pathogenesis of virulent MDV lacking the cytoplasmic tail of pUL49.5

Our initial studies utilized the BAC20-derived virus (v20, 1084), which is based on attenuated vaccine strain 584p80C, using *in vitro* assays to examine the role of the cytoplasmic tail of MDV pUL49.5 in down-regulation of MHC class I surface expression. In order to identify the contribution of the pUL49.5 cytoplasmic tail during MDV pathogenesis *in vivo*, recombinant viruses were generated using the infectious pRB-1B clone (Petherbridge et al., 2004). A mutant BAC was generated with a deletion of the UL49.5 cytoplasmic tail (Table 1, 1157). In addition, a revertant virus was constructed in which the deletion of the cytoplasmic tail was restored (Table 1, 1184). Consistent with the findings of the v20 $\Delta$ 49.5tail mutant viruses (v1130 and v1131), there was no growth defect of the v1157 *in vitro* based on plaque size and multi-step growth kinetics compared to its revertant (v1184) and the parental vRB-1B (v1085) (data not shown).



**Fig. 2.** Down-regulation of MHC class I surface expression by MDV pUL49.5. (A–C) Flow cytometry analysis of RP9 cells transiently transfected with pcUL49.5 (A), pcUL49.5Δtail (B), or pcUL49.5Δtail (C). MHC class I surface expression following transfection with expression constructs were compared to RP9 cells transfected with the empty vector (pcDNA3.1). (D–F) Two RP9 cell lines stably expressing either classical (BF2\*21) or non-classical (BF1\*21) FLAG-tagged MHC class I glycoproteins (Hunt et al., 2001) were transiently transfected with pcDNA3.1 or pcDNA-UL49.5 and analyzed by flow cytometry for classical (D) and non-classical MHC class I (E), as well as MHC class II (F) surface expression. Red lines indicate cells expressing MDV pUL49.5 using anti-FLAG antibodies, black lines indicate cells harboring vector only, and dotted curves indicate antibody isotype controls.

In a first animal trial, the development of MD was examined over an 8-week period in MD-susceptible P2a chickens infected with v1085 (parental vRB-1B), v1157 (Δ49.5tail), or v1184 (Δ49.5tail-rev). By the termination of the experiment, 85% of chickens infected with the deletion mutant v1157 developed MD, while 93% and 73% of chickens infected with v1085 and v1184, respectively, developed MD. Back titration of viral stocks used in this experiment showed chickens were infected with 850, 3,000, and only 5 PFU/chicken for v1085, v1157, and v1184, respectively, which might explain the slightly lower MD incidence in the v1184 group.

In a second animal trial, both viral replication and the development of MD were examined. Analysis of viral replication by quantitative real-time PCR of viral loads in peripheral blood showed there were no significant differences among the three viruses (Fig. 4A). We were unable to sample chickens beyond 4 weeks after infection since our sample size of chickens for analysis was reduced substantially because of losses due to MD (Fig. 4B). In this experiment, the v1157 deletion virus induced slightly reduced incidence (82%) compared to 100% for

v1085 (parental) and the 1157 revertant (v1184). The observations of a slightly reduced oncogenic potential of the RB-1B mutant lacking the cytoplasmic tail of the UL49.5 protein were similar to the observations made in the first animal experiment. The differences, however, were not statistically significant and only represent a trend.

Another important aspect of MDV pathogenesis is that virus is capable of spreading from chicken-to-chicken by horizontal transmission after being shed from the skin of infected chickens. As discovered earlier, recombinant virus derived from the original pRB-1B infectious BAC clone was incapable of horizontal spread; therefore, we were unable to examine this aspect of MDV pathogenesis in the first two trials. Recently, horizontal transmission was restored in viruses derived from pRB-1B BAC in which the gC, gD, and UL13 ORFs containing frame-shift mutations were repaired (Jarosinski et al., 2007). Using the restored virus, we again deleted the MDV UL49.5 cytoplasmic tail and also generated a revertant virus in which the cytoplasmic tail was restored (Table 1). In a third animal trial evaluating viral replication in peripheral blood, MD incidence, and

**Table 1**  
Designation of virus by number.

Number <sup>a</sup>	Originating clone <sup>b</sup>	Modifications <sup>c</sup>	Spread <sup>d</sup>
1084	BAC20 (Schumacher et al., 2000; Spatz et al., 2008)	Multiple (Spatz et al., 2008)	—
1130	1084 (BAC20)	ΔUL49.5cytoplasmic tail	—
1131	1084 (BAC20)	ΔUL49.5cytoplasmic tail	—
1085	pRB-1B with US2 restored (Petherbridge et al., 2004; Spatz et al., 2007; Jarosinski et al., 2007)	US2-restored (Jarosinski et al., 2007)	— <sup>e</sup>
1157	1085 (pRB-1B)	US2-restored; ΔUL49.5 cytoplasmic tail	— <sup>f</sup>
1184	1157	Revertant of 1157	— <sup>f</sup>
1269	None (Jarosinski et al., 2007)	US2-restored; UL13-, gC-, and gD-repaired	+ <sup>e</sup>
1360	1269	US2-restored; UL13-, gC-, and gD-repaired; ΔUL49.5 cytoplasmic tai	+ <sup>f</sup>
1407	1360	Revertant of 1360	+ <sup>f</sup>

<sup>a</sup> Designated number based on the bacterial artificial chromosomes stock number.

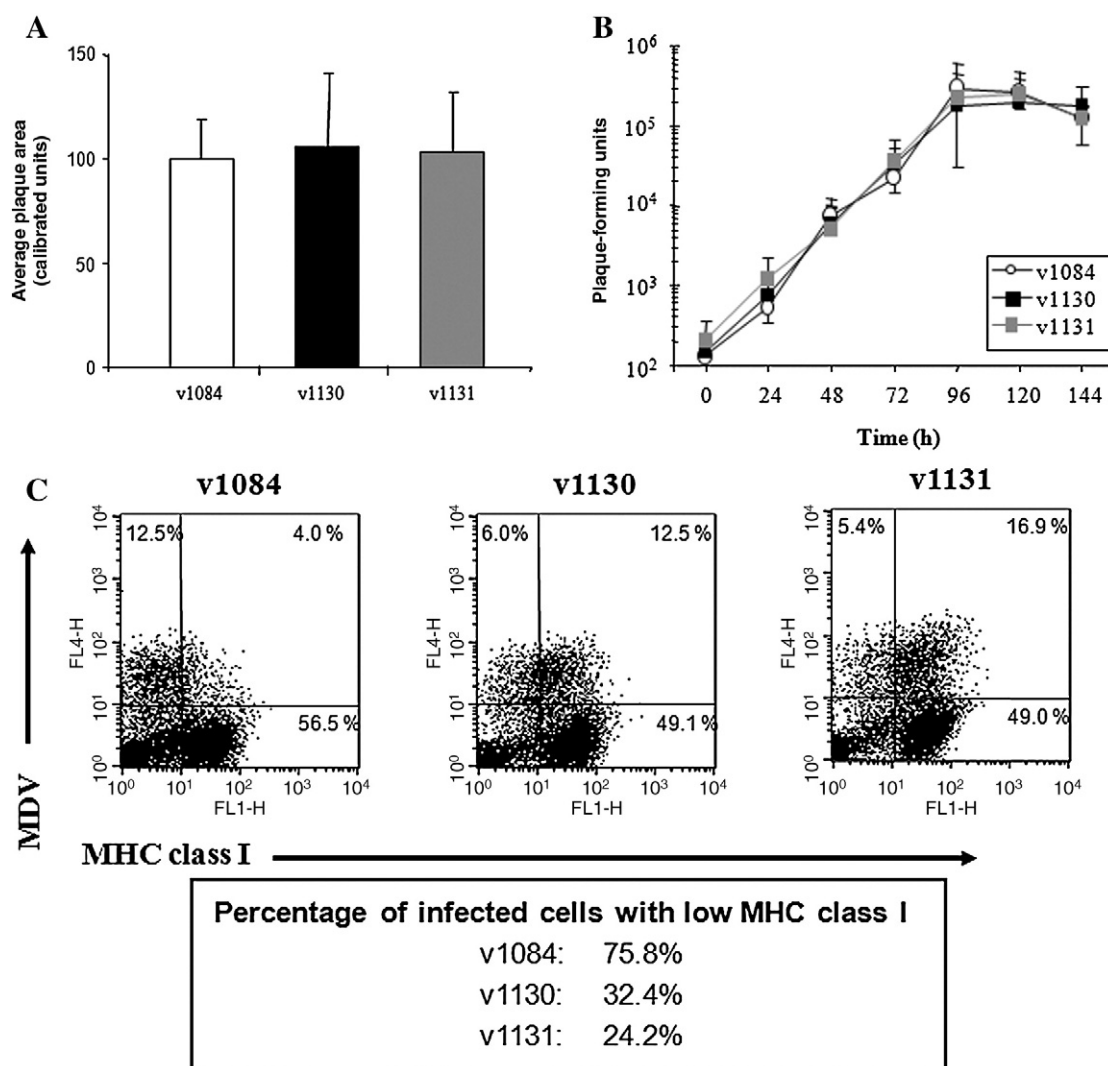
<sup>b</sup> Originating BAC clone that mutations were done in.

<sup>c</sup> Modifications compared to the published sequence of Md5 (Tulman et al., 2000).

<sup>d</sup> Indicates whether the virus horizontally spread to sentinel chickens.

<sup>e</sup> Previously published (Jarosinski et al., 2007).

<sup>f</sup> Determined in this study.



**Fig. 3.** Plaque size, growth kinetics, and down-regulation of MHC class I surface expression of MDV lacking the UL49.5 cytoplasmic tail. (A) Plaque sizes were measured at 5 days pi as previously described. No difference was seen between parental (v1084) and two viruses lacking the UL49.5 cytoplasmic tail (v1130 and v1131). (B) Growth kinetics of v1084, v1130, and v1131 showing no differences between all three viruses. (C) OU2 cells were analyzed by double immunofluorescent staining and FACS analysis at 72 h after co-seeding of infected with uninfected cells as detailed in the materials and methods. The X-axis (FL-1) represents MHC class I surface expression and the Y-axis (FL-4) represents MDV-specific reactivity. The percentage of infected cells with low MHC class I expression was calculated according to the formula:  $UL / (UL + UR) \times 100$ , where UL is the number of cells in the upper left quadrant (MDV<sup>high</sup>MHC<sup>low</sup>) and UR is the number of cells in the upper right quadrant (MDV<sup>high</sup>MHC<sup>high</sup>).

horizontal transmission, we again found little difference between the v1269 (parental virus that is spread competent), v1360 ( $\Delta$ 49.5tail), and v1407 ( $\Delta$ 49.5tail-rev) as both mutant and revertant viruses replicated to similar levels (Fig. 5A), induced 100% MD incidence by 12 weeks (Fig. 5B), and horizontally spread from chicken-to-chickens with 56% and 88% of contact chickens, respectively, developing MD at the time of termination (12 weeks). From these results, we concluded that down-regulation of MHC class I by the MDV UL49.5 gene product is not a prominent virulence mechanism, at least in highly susceptible B<sup>19</sup>B<sup>19</sup> chickens.

In a final chicken experiment, we examined if the UL49.5 cytoplasmic tail played a role dependent on the MHC haplotype of the chicken. MD-resistant N2a chickens (B<sup>21</sup>B<sup>21</sup> haplotype) were infected with v1360 or its revertant (v1407), and viral replication, MD incidence, and horizontal transmission were measured (Fig. 6). Early replication (<10 dpi) of v1360 and v1407 were indistinguishable, whereas there appeared to be a divergence in that there was a rapid decrease in replication in the v1360 group, followed by an increase that was maintained until monitoring of replication was stopped (Fig. 6A). In contrast, the  $\Delta$ 49.5tail-rev group had a slower decrease in replication from 10 to 21 days pi, which then increased modestly up

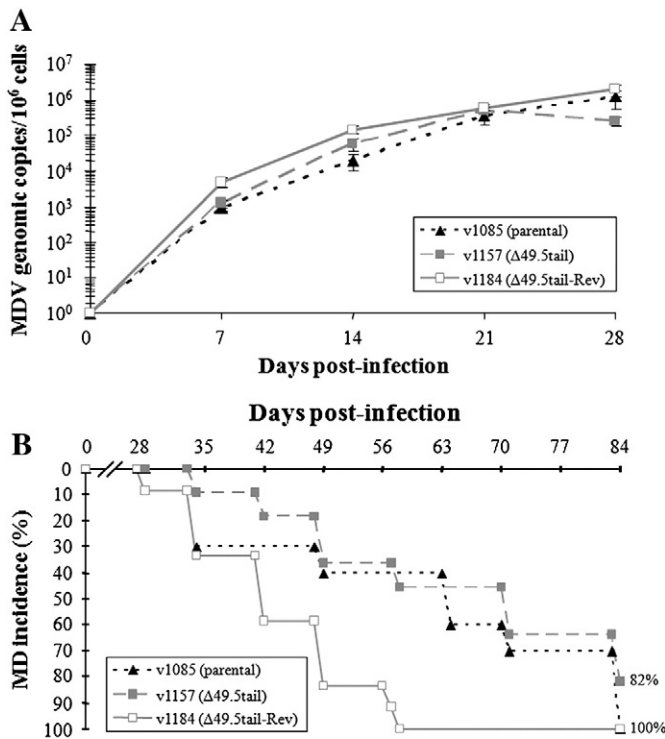
to 35 days pi. There appeared to be a difference in MD incidence and horizontal transmission between the two groups as well with the  $\Delta$ 49.5tail group causing less MD (60%) compared to its revertant group (87%) and this was also reflected in the MD incidence in contact chickens (17% to 33%, respectively).

## Discussion

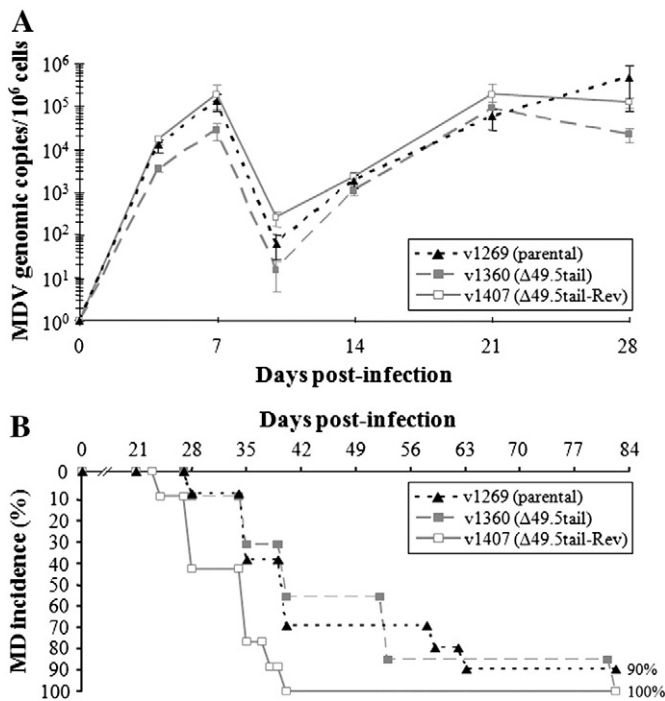
It was previously shown that MDV can down-regulate MHC class I surface expression (Hunt et al., 2001). In this report, we show that the MDV encoded pUL49.5, more specifically its cytoplasmic tail, is directly responsible for down-regulation of MHC class I surface expression. We also were able to show that MDV pUL49.5 can induce this effect in the absence of other viral proteins. Clearly, transfection of chicken RP9 B cells with a plasmid expressing FLAG-tagged MDV pUL49.5 down-regulated MHC class I surface expression in cells expressing pUL49.5 (Fig. 2A), while the HSV-1 UL49.5 expression did not (Fig. 2B). Deletion of the cytoplasmic tail of MDV pUL49.5 showed that this region was essential for MHC class I surface down-regulation (Fig. 2C).

In the context of viral infection, the avirulent BAC20 virus was able to down-regulate MHC class I surface expression following infection

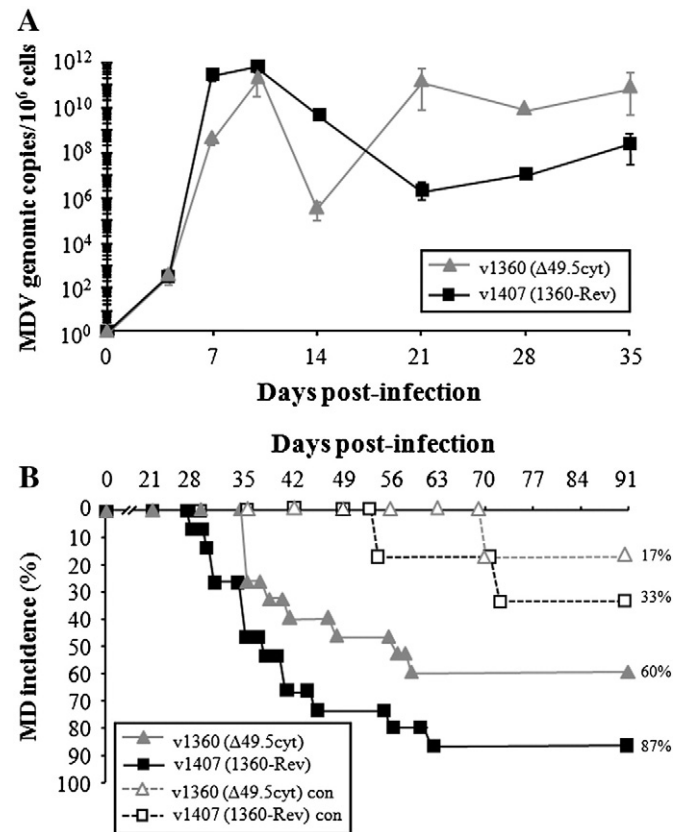




**Fig. 4.** MDV DNA replication and MD incidence of virus lacking the 49.5 cytoplasmic tail. (A) MDV genomic copies were quantified in the whole blood of infected chickens at 7, 14, 21, and 28 days pi by detection of ICP4 DNA for parental (v1085), Δ49.5tail (v1157), and the Δ49.5tail revertant (v1184) and normalized to the chicken iNOS gene for DNA loading using qPCR assays. Standard error of the mean (SEM) bars for each group are shown. (B) Percent of MD incidence was determined for each group during the 12-week evaluation period.



**Fig. 5.** MDV DNA replication and MDV incidence of virus lacking the 49.5 cytoplasmic tail (Δ49.5tail) that can horizontally spread in highly MD-susceptible P2a chickens. (A) MDV genomic copies were quantified in the whole blood of parental (v1269)-, Δ49.5tail (v1360)-, and the Δ49.5tail revertant (v1407)-infected chickens at 4, 7, 10, 14, 21, and 28 days pi as in Fig. 4. SEM bars for each group are shown. (B) Percent of MD incidence was determined for each group during the 12-week evaluation period.



**Fig. 6.** MDV DNA replication and MDV incidence of virus lacking the 49.5 cytoplasmic tail that can horizontally spread in MD-resistant N2a chickens. (A) MDV genomic copies were quantified in the whole blood of infected chickens at 4, 7, 10, 14, 21, and 28 days pi as in Fig. 4 for v1360 and v1407. SEM bars for each group are shown. (B) Percent of MD incidence was determined for each group during the 13-week evaluation period.

of the immortalized OU2 chicken fibroblast cell line that constitutively expresses high levels of MHC class I on its surface. However, when the cytoplasmic tail of pUL49.5 was removed in two independent viruses, the level of MHC class I down-regulation was reduced considerably (Fig. 3C), results that were consistent with the findings in the pUL49.5Δtail expression experiments (Fig. 2).

In order to examine the importance of the MDV pUL49.5 cytoplasmic tail during MDV pathogenesis, we created UL49.5Δtail mutants based on an infectious clone of the highly virulent RB-1B strain (pRB-1B). In these studies, we found no difference between wild-type and revertant viruses compared to viruses lacking the MDV pUL49.5 cytoplasmic tail after infection of chickens of an MHC haplotype (B<sup>19</sup>B<sup>19</sup>) that is associated with increased susceptibility to MDV infection (Cole, 1968). In contrast, MDV lacking the cytoplasmic tail of pUL49.5 were less pathogenic in chickens homozygous for the B<sup>21</sup> MHC allele, suggesting an MHC haplotype-specific effect of MHC class I down-regulation in the overall pathogenesis of virulent MDV. Unfortunately, we were unable to directly examine the down-regulation affect of vRB-1B *in vitro* since virulent MDV is selective for which cell types it will efficiently infect *in vitro*. In multiple attempts, we were unable to establish infection in OU2 cells with vRB-1B (Jarosinski and Osterrieder, unpublished observations). In addition to the inability of vRB-1B to infect OU2 cells, all cell types in which propagation of virulent MDV is possible, such as CEC, chicken kidney cell (CKC), or duck embryo fibroblast (DEF) cultures, do not express significant levels of MHC class I on the cell surface (Jarosinski and Osterrieder, unpublished observations). Additionally, levels of surface MHC class I on CKC or CEC cultures treated with interferon did not increase significantly enough to show down-regulation with the vRB-1B lacking the cytoplasmic tail of pUL49.5. Thus, due to technical

limitations we were unable to test MDV pUL49.5 MHC class I down-regulation properties in the context of a virulent virus *in vitro*.

At present, we surmise that MDV pUL49.5 down-regulates MHC class I surface expression on infected cells by blocking peptide transport by TAP based on results of studies using MDV UL49.5 homologs including BHV-1 (UL49.5), EHV-1 (gene 10), and PRV (gN) (Koppers-Lalic et al., 2005). In preliminary experiments, however, we were not able to demonstrate degradation of TAP, which would hint that MDV pUL49.5 acts similarly to its EHV-1 counterpart that blocks peptide transport but does not result in TAP degradation (Koppers-Lalic et al., 2005). In this context it is important to note that the close relative of MDV, varicella-zoster virus (VZV), also down-regulates MHC class I expression, but interestingly the VZV UL49.5 homolog, ORF 9A, does not mediate this effect although binding of the ORF 9A product to TAP still is observed (Koppers-Lalic et al., 2005). It was recently shown that the serine/threonine protein kinase ORF66 (HSV US3 homolog) is able to mediate MHC class I down-regulation; however, other VZV proteins are most likely able to down-regulate MHC class I as well since VZV lacking ORF66 kinase domain still is capable of down-regulating MHC class I expression (Eisfeld et al., 2007). It is unknown at this time if MDV pUS3 is able to down-regulate MHC class I surface expression or whether this activity is possibly conferred by other MDV proteins. *In vitro* experiments with the avirulent BAC20 strain suggest that although mutants lacking the pUL49.5 cytoplasmic tail fail to down-regulate MHC class I surface expression as efficiently as the parental virus (Fig. 3), there are still 25–35% infected cells that still maintain low levels of MHC class I surface expression. These data indicate that (an)other gene product(s) may also have MHC class I down-regulating activity and may be important for complete MHC class I surface expression down-regulation *in vitro*. This interpretation would also explain the lack of significant pathogenic changes with the virulent MDV lacking the pUL49.5 cytoplasmic tail (Figs. 4–6) and be consistent with the findings in murine cytomegalovirus (MCMV) immune evasion where a clear reduction in virulence was only shown in the absence of a whole set of genes involved in MHC class I down-regulation (Pinto et al., 2006).

In summary, we have shown that the MDV UL49.5 gene product down-regulates MHC class I surface expression *in vitro* in chicken fibroblast and B cells. This mechanism, however, had little effect *in vivo* suggesting that other proteins may be involved during infection in the chicken. Based on studies addressing pUL49.5 action in related viruses, our future studies will focus on the exact mechanism by which the MDV ortholog confers this activity and will first concentrate on the putative TAP interaction in the process. Additionally, we are evaluating other potential MHC class I down-modulating genes encoded by MDV.

## Materials and Methods

### Cell cultures and viruses

CEC cultures were prepared from 10-day-old specific-pathogen-free (SPF) embryos, while CKC cultures were prepared from 14-day-old SPF chickens (Schat and Purchase, 1998). CHCC-OU2 (OU2) cells are a chemically transformed CEC line and have previously been described (Hunt et al., 2001; Ogura and Fujiwara, 1987). The lymphoblastoid cell line, LSCC-RP9 (RP9) cells are an avian leukosis

virus-transformed cell line ( $B^2B^{15}$ ). Both cell lines were grown in Leibovitz-McCoy medium supplemented with 10% fetal bovine serum (FBS), 12% tryptose phosphate broth, and antibiotics in a humidified atmosphere of 5% CO<sub>2</sub>, although the OU2 cells were grown at 38 °C, while RP9 cells were grown at 41 °C.

Recombinant viruses were reconstituted by transfecting CEC cultures with purified BAC DNA using the CaPO<sub>4</sub> precipitation method (Schumacher et al., 2000) with pCAGGS-NLS/Cre, a plasmid expressing the Cre enzyme for excision of mini-F sequences using loxP sites (a kind gift from Dr. Michael I. Kotlikoff, Cornell University, Ithaca, NY), as previously described (Jarosinski et al., 2007). For propagation of reconstituted viruses, infected CEC cultures were passed onto CKC cultures after the development of plaques following transfection and expanded. Recombinant virus stocks were examined for the removal of mini-F vector sequences as previously described (Jarosinski et al., 2007) and shown to lack the mini-F vector (data not shown). BAC clones generated are shown in Table 1 with genome modifications indicated.

### UL49.5 expression vectors and antibodies

To investigate the effects of MDV UL49.5 on MHC class I expression, the UL49.5 gene was PCR amplified and cloned into the *HindIII/XbaI* sites of the pcDNA3.1/Zeo (Invitrogen, Carlsbad, CA) expression vector (pcDNA-UL49.5). The UL49.5 insert in the expression plasmid was DNA sequenced (BigDye® Terminator v3.1 Cycle Sequencing Kit, Life Technologies, Carlsbad, CA) to assure integrity of the sequence. The pcDNA-UL49.5 was used for transient expression in RP9 chicken B cell lines stably expressing either classical (BF2\*21) or non-classical (BF1\*21) FLAG-tagged MHC class I glycoproteins (Hunt et al., 2001). Control or pcDNA-UL49.5 plasmids were transfected into  $1 \times 10^7$  RP9 cells with the Nucleofector II electroporation device from Amaxa (Lonza Walkersville Inc., Walkersville, MD) using solution V and program T-001. The classical and non-classical MHC class I expression was analyzed by flow cytometry (BD FACSCalibur, San Jose, CA) using either the C6B12 antibody specific to chicken MHC class I (DSHB, University of Iowa, Iowa City, Iowa) or the M1 anti-FLAG monoclonal antibody (Sigma Chemical Company, St. Louis, MO) and detected with fluorescein isothiocyanate-labeled goat anti mouse (Cappel, Durham, NC) 24 and 48 h after transfection.

### Construction of 49.5Δtail recombinant MDV

Mutagenesis was performed as using two-step Red recombination exactly as previously described (Jarosinski et al., 2007) with primers shown in Table 2. All final clones were screened by restriction fragment length polymorphism, Southern blot, and nucleotide sequencing analyses for confirmation purposes.

### Blood DNA extraction and qPCR

DNA was extracted from whole blood for use in qPCR exactly as previously described (Jarosinski et al., 2007) using the DNeasy® 96 Tissue Kit from Qiagen, Inc. (Valencia, CA). Quantification of MDV genomic copies using qPCR was also performed exactly as previously described (Jarosinski et al., 2007) using primers, and probe specific for the MDV ICP4 was used in qPCR assays. All qPCR assays were performed

**Table 2**  
Primers used for modification of the Marek's disease virus UL49.5 gene.

Modification	Direction <sup>a</sup>	Sequence <sup>b</sup>
Δ49.5tail	Forward	tctccgccccttagcgggttcgtatcacgcatgcttttaattatgtggcggtgtagggataacagggtaatcgattt
	Reverse	gaagagataagagatttgcacgcccacataatttaaaagcatgcgtgatacgaacggcagtggttacaaccaattaacc
Δ49.5tail-rev	Forward	ctcttagcgggttcgtatcacgcatgctttcgaacttaccgcagatatgtttaagaggagtggttagggataacagggtaatcgattt
	Reverse	ataaggattgtccacgcccacataatttaaccactcctctttaaacatatctgcggtgaatagtcggccagtggttacaaccaattaacc

<sup>a</sup> Directionality of the primer.

<sup>b</sup> Underlined sequence indicates the template binding region of the primers for PCR amplification with pEPKanS. Bold indicates the insertion of the cytoplasmic tail nucleotide sequence into the Δ49.5 mutant.

in an ABI Prism 7500 Fast Real-time PCR System (Applied Biosystems, Inc.) and the results were analyzed using Sequence Detection Systems version 1.4 software supplied by the manufacturer. Using the standard curve generated for each gene, the number of copies for *ICP4* and *iNOS* was determined by using the  $C_T$  value for that sample.

#### Multi-step growth kinetics and measurement of plaque areas

Multi-step growth kinetic analysis was determined as previously described (Dorange et al., 2002). Briefly,  $1 \times 10^6$  CEC were infected with 100 PFU of each respective virus, and at various times following infection, infected cells were trypsinized and plated onto fresh CEC at serial 10-fold dilutions in triplicate. After 5 days pi, cells were fixed with 90% ice-cold acetone and indirect immunofluorescence using polyclonal anti-MDV chicken sera and secondary goat anti-chicken IgG labeled with Alexa Fluor® 488 (Invitrogen, Inc.) was used to visualize plaques as previously described (Jarosinski et al., 2005).

Plaque areas were measured exactly as previously described (Jarosinski et al., 2005) using The National Institutes of Health ImageJ software ([rsb.info.nih.gov/ij/](http://rsb.info.nih.gov/ij/)) and means with standard deviations were determined for each virus.

#### Experimental infection of chickens

SPF P2a ( $B^{19}B^{19}$ ) or N2a ( $B^{21}B^{21}$ ) chickens were inoculated intra-abdominally with 2,000 PFU of the various viruses at 1 day of age. Where indicated, sentinel chickens were housed together with infected birds to determine horizontal spread of virus. Chickens were evaluated daily for symptoms of MD, euthanized, and examined for gross lesions when birds showed clinical evidence of MD. Chickens were obtained from departmental flocks, housed in isolation units, and water and food were provided *ad libitum*. All experimental procedures were conducted in compliance with approved Institutional Animal Care and Use Committee (IACUC) protocols. Chickens were assigned to treatment groups using a randomization table.

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